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LETTER

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Contrasting levels of β-diversity and underlying phylogenetic trends indicate different paths to chemical diversity in highland and lowland willow species

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Abstract

Diverse specialised metabolites contributed to the success of vascular plants in colonising most terrestrial habitats. Understanding how distinct aspects of chemical diversity arise through heterogeneous environmental pressures can help us understand the effects of abiotic and biotic stress on plant evolution and community assembly. We examined highland and lowland willow species within a phylogenetic framework to test for trends in their chemical α-diversity (richness) and β-diversity (variation among species sympatric in elevation). We show that differences in chemistry among willows growing at different elevations occur mainly through shifts in chemical β-diversity and due to convergence or divergence among species sharing their elevation level. We also detect contrasting phylogenetic trends in concentration and α-diversity of metabolites in highland and lowland willow species. The resulting elevational patterns contribute to the chemical diversity of willows and suggest that variable selective pressure across ecological gradients may, more generally, underpin complex changes in plant chemistry.

KEYWORDS
divergence, elevation, escalation, flavonoids, herbivory, salicinoids, *Salix*, specialised metabolites

INTRODUCTION

A broad array of metabolites has contributed to the success of vascular plants, allowing them to colonise various habitats and occupy distinct niches (Pellissier et al., 2018; Wetzel & Whitehead, 2020). Diverse specialised metabolites serve plants as defences against herbivores or protection from adverse abiotic conditions (Defossez et al., 2018; Volf et al., 2015, 2018). Understanding how chemical diversity arises through heterogeneous environmental pressures can help us unravel the effects of abiotic and biotic stress on plant evolution and community assembly (Maron et al., 2019; Sedio et al., 2012). This is especially important in our changing world where selective pressures on plants are shifting rapidly (Pellissier et al., 2018).

Plants face myriad selective pressures in natural environments. When searching for the mechanisms driving diversity of plant specialised chemistry, we need to understand how the prevailing selective landscape shapes trends in metabolite concentration, α-diversity (e.g. the number of metabolites in a plant) and β-diversity (chemical variation among plants). We can build on a large body of previous work relating functional traits in plant communities to habitat characteristics in a phylogenetic context (e.g. Cavender-Bares et al., 2004; Sedio et al., 2012; Webb et al., 2002). Abiotic pressures should filter species that can persist within a community based on their traits, resulting in functional similarity across species (Cavender-Bares et al., 2004; Sedio et al., 2012). In contrast, antagonistic biotic interactions can prevent co-occurrence of species with functionally similar traits and defences (Becerra, 2007).

In terms of specialised chemistry, shared abiotic pressure in plant communities should support directional phylogenetic trends towards increased content of metabolites critical for plant survival, while reducing their β-diversity among species (Defossez et al., 2021; Volf et al., 2022). In contrast, chemical β-diversity should be high in communities exposed to strong biotic pressures, such as herbivory, that generate negative density-dependent feedback on assemblages of species sharing defensive traits (Sedio & Ostling, 2013). Evidence suggests that there is competition for enemy-free space among regionally co-occurring plants, promoting their chemical divergence, which limits the number of herbivores they share (Becerra, 2007; Kursar et al., 2009; Volf et al., 2019). Additionally, the fitness costs inflicted by insect herbivores are thought to support phylogenetic escalation in α-diversity of chemical defences, allowing derived plant species with novel defences to escape herbivores over macroevolutionary timescales or to defend themselves against multiple natural enemies (Ehrlich & Raven, 1964; Maron et al., 2019; Whitehead et al., 2021).

Plants growing along elevational gradients represent ideal systems for dissecting the environmental drivers of chemical diversity (Defossez et al., 2018; Moreira et al., 2018). Abiotic stress increases with elevation, promoting investment into metabolites that are involved in protecting plants against UV-irradiation and serve as antioxidants (Volf et al., 2022). In contrast, herbivory generally declines with elevation resulting in lower biotic pressure on high-elevation plants (Pellissier et al., 2014; Sam et al., 2019; Volf et al., 2022). In addition to driving trends in the presence and concentration of metabolites, elevational clines in environmental stress also have the potential to drive corresponding trends in their structural diversity (Volf et al., 2022). Structurally unrelated metabolites are synthesised through different metabolic pathways, and they are likely to serve different functions (Philbin et al., 2022). Using structurally informed indices can thus reveal functional trends in plant chemistry even in situations in which simple indices, such as total content of broad classes of metabolites, fail to show any patterns (Moles et al., 2011; Sedio et al., 2017).

Here we test if chemical diversity shows differential trends among species that regionally co-occur at different elevations ('realised habitats' sensu Webb et al. (2002)) using willows growing in the Austrian Alps and adjacent lowland regions. Willows (*Salix* L.) colonised the Alps repeatedly, with some of the colonisation occurring recently during the last glacial periods (Wagner et al., 2021). They form diverse local assemblages and are broadly distributed from lowland floodplains to the alpine zone. Willow specialised metabolites mainly consist of phenolics with distinct functions, facilitating the comparison of trends in metabolites that play different roles in plant biology (Volf et al., 2015, 2022). The phenolics found in willows include numerous flavonoids that are primarily involved in protecting willows against abiotic stress (Tegelberg & Julkunen-Tiitto, 2001). Additionally, willows possess various tannins and salicinoids that defend them from insect herbivores and fungal pathogens (Hjalten et al., 2007; Volf et al., 2015). Salicinoids in particular have been studied for their anti-herbivore roles as they can deter generalist insects from feeding. In contrast, specialised herbivores can use them as feeding or oviposition cues (Denno et al., 1990; Hjalten et al., 2007).

Theory and experimental work suggest that lowland plants experience higher rates of herbivory while highland species experience less favourable abiotic conditions. We made three predictions based on this expectation. (i) Leaves from highland willow species will have a greater concentration of secondary metabolites. This trend will be especially pronounced in metabolites such as flavonoids that serve as protection against abiotic conditions. (ii) In contrast, lowland willows will exhibit greater α- and β-diversity in their chemistry. Due to the recent, multiple independent colonisation events of the Alps by different willow species (Wagner et al., 2021), we might not be able to detect elevational differences because of confounding phylogenetic signal. However, we should still detect non-random autocorrelation in traits by using methods sensitive to phylogenetic

trends. Hence, our final prediction is related to, but not contingent upon, the first two. Specifically, we predict that (iii) lowland species will show directional phylogenetic trends towards higher α-diversity in their metabolites and generally more divergent chemical profiles than their highland relatives while highland species will show directional trends towards higher concentration and activity of metabolites.

Overall, we show that differences in chemistry among willows growing at different elevations occur mainly due to shifts in chemical β-diversity among species sympatric in elevational range. We also detect contrasting phylogenetic trends in concentration and α-diversity of metabolites among the lowland versus highland species. This suggests that different evolutionary trajectories may act even in recently assembled plant communities experiencing different sources of stress.

MATERIALS AND METHODS

Field sampling

We studied 29 species of willows in the Czech Republic and Austria, encompassing an elevation gradient spanning >2500 m (175–2620 m asl). The sampling covers the majority of the regionally occurring 33 species (Table S1) and excludes mainly those occurring at mid elevations (Wagner et al., 2021). We also sampled *Populus tremula* as an outgroup. We divided the willow species into two categories based on their typical elevational range and sampled four to five individuals per species in the typical zone of their occurrence (Table S2). As 'highland' species, we classified 14 species that occur in the subalpine and alpine zones but not in the colline zone (Wagner et al., 2021). As 'lowland' species, we classified 15 species that primarily occur in the colline and montane zones. Three of the 'lowland' species (*Salix caprea*, *S. pentandra* and *S. purpurea*) that typically occur in the colline and montane zones also include populations occurring in the subalpine zone. The intraspecific elevational variation in concentration or α-diversity of willow metabolites is typically smaller than interspecific differences and should not obscure the patterns explored here (Volf et al., 2022). Nonetheless, we limited our sampling of these three species to populations from the colline zone only. To broaden the phylogenetic sampling, we also included *S. acutifolia* that is native to Eastern Europe and sampled it at the elevation typical for this species in its natural range. We extracted annual daily mean temperature and annual precipitation amount from CHELSA V1. 2 database (Karger & Zimmermann, 2019) for all individuals from lowland and highland species based on their GPS coordinates. The two groups differed in both temperature ($f^2 = 16.422$, $F_{(1,27)} = 443.2$, $p < 0.001$) and precipitation ($f^2 = 5.489$, $F_{(1,27)} = 148.2$, $p < 0.001$) when compared with one-way ANOVA using species means.

We sampled freshly matured leaves from the central part of the shoot from each individual in May–July 2020, accounting for differences in leaf phenology across elevations. We used 20 undamaged leaves (or up to 100 leaves in the case of *S. serpyllifolia* with extremely small leaves) for chemical analyses detailed below. We haphazardly sampled additional 50 leaves to measure insect herbivory. We photographed the leaves and quantified herbivory in ImageJ (Abràmoff et al., 2004).

Chemical analysis

The leaves sampled for chemical analyses were stored in liquid nitrogen in the field. Later, we freeze-dried them, removed the petioles and homogenised the leaves. First, we performed broad-spectrum, untargeted metabolomics to screen for smaller metabolites and to provide information on metabolite richness and structural α- and β-diversity. We supplemented this analysis with quantifying the concentration and composition of proanthocyanidins that represent the major group of tannins in willows (Volf et al., 2022). Finally, we ran two activity assays to quantify two major functions of polyphenols in anti-herbivore defence—oxidative activity and protein precipitation capacity.

Untargeted metabolomics

Small organic molecules were extracted from ca. 10 mg (in 0.01 mg accuracy) of homogenised material using methanol/water (90:10, v/v) following Sedio et al. (2021). We optimised UHPLC–MS parameters to detect and fragment metabolites representing a wide range in polarity and mass (Sedio, Boya, et al., 2018). Separation of metabolites by UHPLC was followed by heated electrospray ionisation (HESI) in positive mode using full scan MS1 and data-dependent acquisition of MS2 (dd-MS2) on a Thermo Fisher Scientific QExactive hybrid quadrupole-orbitrap mass spectrometer.

We first calculated metabolite concentrations (peak areas/mg), simple richness (the number of metabolites per sample) and structural β-diversity among the samples. To do so, we aligned chromatograms using Mzmine2 (Pluskal et al., 2010), inferred molecular formulae using Sirius (Dührkop et al., 2019), predicted structures using CSI:fingerID (Dührkop et al., 2015) and classified the metabolites using CANOPUS (Dührkop et al., 2021). Using this information, we created three datasets including (i) the whole metabolome (all detected metabolites), (ii) flavonoids and (iii) salicinoids plus their derivatives. We then uploaded the data to the Global Natural Products Social (GNPS) Molecular Networking platform (Wang et al., 2016) and used them to generate a molecular network using the 'feature-based molecular networking' method (Nothias et al., 2020) as described

in Sedio et al. (2021). We used the output to calculate the chemical structural-compositional similarity (CSCS) metric for every pair of samples in the three datasets to quantify the structural variation among samples based on MS/MS spectral similarity of the metabolites they contain following Sedio et al. (2017). The CSCS metric provides a chemically meaningful measure of structural variation in metabolite composition even in cases in which samples do not share a metabolite. Structurally similar, but unshared metabolites would contribute zero to indices based solely on presence or concentration of shared metabolites (e.g. Bray–Curtis similarity) but make a positive contribution to CSCS. We calculated two measures of structural β-diversity based on the CSCS matrices: (i) CSCS distance among every pair of samples and (ii) uniqueness measured as mean pairwise CSCS distance between the given sample and all other samples from the same elevation category.

Second, we analysed the data with Qemistree (Tripathi et al., 2021) to infer structural α-diversity of metabolites in the samples. We simultaneously inferred molecular structures using Sirius (Dührkop et al., 2019) and CSI:FingerID (Dührkop et al., 2015) and classified metabolites using ClassyFire. For each of the three metabolite datasets, we built hierarchical dendrograms that reflect the structural similarity among individual metabolites and transformed these into distance matrices (Tripathi et al., 2021). We used these matrices to calculate mean pairwise distance (MPD) index for the whole metabolome, flavonoids and salicinoids within a sample using NRI.p function in iCAMP package (Ning et al., 2020). We used MPD indices reflecting mean structural similarity among metabolites within a sample as our measure of structural α-diversity of metabolites. Please see Appendix S1 for details.

Proanthocyanidin quantification

We extracted proanthocyanidins from ca 20 mg (in 0.01 mg accuracy) of homogenised material using acetone/water (80:20, v/v) solvent as described in detail in Malisch et al. (2016). We quantified procyanidin and prodelphinidin units found in proanthocyanidins (in mg/g) by UHPLC-QqQ-MS/MS with the methods of Engström et al. (2014, 2015) as described in, for example, Malisch et al. (2016). We used purified procyanidin-rich proanthocyanidin fraction (procyanidin units) and purified prodelphinidin-rich proanthocyanidin fraction (prodelphinidin units) as external standards to quantify the focal compounds.

Polyphenol activities

We measured polyphenol oxidative activity following Salminen and Karonen (2011) using gallic acid as the standard. We measured protein precipitation capacity with the turbidimetric well-plate assay (Engström et al., 2019) using pentagalloylglucose as the standard. Both assays gave activities in mg/g dry weight.

Willow phylogeny

We used whole-genome sequencing to sequence 35 willow species representing all major clades in previous phylogenomic trees and all the species used in our chemical analyses (He et al., 2021; Wagner et al., 2020). In addition, we included *Populus tremula* as an outgroup and *Salix purpurea* from NCBI (Zhou et al., 2020) (Table S3). Total genomic DNA was extracted from silica gel-dried leaves using the MagAttract HMW DNA kit (Qiagen). Whole-genome library preparation and DNA sequencing in an Illumina NovaSeq 6000 platform producing 2 × 150bp paired end reads were carried out by the company Novogene. Please see Appendix S1 for the details on quality-check, assembly, mapping and alignments.

A concatenation-based phylogenetic tree was inferred using a maximum-likelihood inference (ML) in IQ-TREE version 2.1.2 (Minh et al., 2020). Branch support was evaluated with 1000 likelihood bootstrap replicates. Using the pruned phylogenetic tree that contained only the focal willow species (Figure S1), we created an ultrametric tree by converting branch lengths to substitution rates and estimating lambda using Penalized Likelihood under a relaxed clock model (Paradis & Schliep, 2019).

Statistical analyses

We first compared arcsine-transformed species means of herbivory damage between lowland and highland willows with one-way ANOVA in R 4.2.0 (R Core Team, 2022). We then compared the trait values among highland lowland species to test our first and second hypothesis. We calculated species means (Table S4) of metabolite concentration, activity, simple richness, structural α-diversity and uniqueness (Table 1). We compared them between lowland and highland species using the ultrametric phylogenetic tree and Phylogenetic Least Squares Regression (PGLS) using the R packages 'nlme' (Pinheiro et al., 2019) and 'phytools' (Revell, 2012). We log-transformed metabolite concentrations and activities. We centred all traits, calculated standardised effect sizes (SES) from models using the transformed data in the R package 'emmeans' (Lenth et al., 2019), and corrected the *p*-values for false discovery rate (Benjamini & Hochberg, 1995).

To further compare structural β-diversity in metabolites among lowland and highland willows, we explored the variation in CSCS distances for all their metabolites detected by untargeted metabolomics, flavonoids and salicinoids with multivariate methods in CANOCO

TABLE 1 Variables that we used to describe willow leaf chemistry.

Chemical variables	Expected trend	Recovered trend
Metabolite concentration and activity		
Quantified as the area under peaks/mg or mg/g of leaf tissue		
Concentration of flavonoids	Higher in highlands	Phylogenetic increase in highlands ***
Concentration of salicinoids	Higher in highlands	***
Concentration of proanthocyanidins (PAs)	Higher in highlands	***
Oxidative activity	Higher in highlands	Phylogenetic increase in highlands
Protein precipitation	Higher in highlands	—
Simple richness		
Number of all metabolites in an individual sample		
Simple richness of whole metabolome	Higher in lowlands	***
Simple richness flavonoids	Higher in lowlands	Phylogenetic increase in highlands
Simple richness salicinoids	Higher in lowlands	***
Structural α-diversity		
Mean pairwise distance (MPD) quantifying the structural relatedness of metabolites in an individual sample		
Structural α-diversity of whole metabolome	Higher in lowlands	—
Structural α-diversity of flavonoids	Higher in lowlands	—
Structural α-diversity of salicinoids	Higher in lowlands	***
Structural β-diversity		
Quantified as distance in CSCS matrices measured with PCoA axes in multivariate analyses or as uniqueness in univariate analyses. Uniqueness was calculated as mean pairwise distance between the given sample and all other samples in CSCS matrices		
CSCS distance in whole metabolome	Higher in lowlands	—
CSCS distance in flavonoids	Higher in lowlands	Higher in lowlands
CSCS distance in salicinoids	Higher in lowlands	—
Uniqueness in whole metabolome	Higher in lowlands	Marginally higher in highlands
Uniqueness in flavonoids	Higher in lowlands	—
Uniqueness in salicinoids	Higher in lowlands	Marginally higher in lowlands

Note: For each variable, we include the expected and recovered differences between highland and lowland species as shown by our analyses after correcting for false discovery rate. The differences were either recovered by direct comparisons of trait values with phylogenetically controlled tests (marked as higher in lowlands or highlands) or suggested by phylogenetic trends and correlation between the distance from the phylogeny root and trait values (marked as phylogenetic increase in highlands). Traits marked with asterisks showed a divergence peak when analysed in DTT plots. Black asterisks indicate traits divergent only in the whole dataset, blue asterisks indicate traits divergent in the whole dataset and in highland dataset and orange asterisks indicate traits divergent in the whole and lowland dataset.

5 (ter Braak & Smilauer, 2012). We first transformed CSCS dissimilarity matrices into a set of axes describing the structural β-diversity among all studied species with Principal Coordinate Analysis (PCoA). Second, we derived a phylogenetic distance matrix based on patristic distances from the concatenation-based tree and transformed it into a set of axes with another PCoA. We selected the phylogenetic PCoA axes with significant effects on chemistry axes with forward selection in Redundancy Analysis (RDA). Finally, we performed partial Principal Component Analysis (pPCA) showing the variation in chemistry while accounting for the effect of phylogeny. To do so, we used the chemistry PCoA axes as response variables and phylogenetic PCoA axes with significant effect on chemistry as covariates. To test if the differences in structural β-diversity were significant, we calculated mean inter-quartile distances on individual pPCA axes among the species sharing their elevation range. We compared the mean distances in the lowland and highland datasets with a paired t-test, weighted with the amount of variation explained by individual pPCA axes using the R package 'weights' (Chambers & Hastie, 2017).

We used three complementary analyses to explore our third hypothesis concerning the phylogenetic trends in chemical traits in highland and lowland willows. We used the same set of traits as in the PGLS analysis. First, we tested for deviations from local phylogenetic means in individual willow traits by calculating the Local Indicator of Phylogenetic Association (local Moran's I) at each tip of the phylogeny. We used patristic phylogenetic distances and tested the significance of each tip value using a two-tailed permutation test with 999 replications using the 'lipaMoran' function in 'phylosignal' (Keck et al., 2016). As we performed the test for multiple traits, we corrected the results for false discovery rate. We then took the upper quartile of largest deviations from the local phylogenetic mean and compared the count of

those towards higher similarity vs. those towards lower similarity between highland and lowland species with a Chi-square test.

Second, we plotted the values of trait disparity through time (DTT) from the phylogeny root to its tips using the function 'dtt' in the R package 'Geiger' (Harmon et al., 2008). We used the average square distance metric to calculate trait disparity and created a null distribution of DTT with 95% confidence intervals using 999 simulations under Brownian motion. We did not apply a correction for performing multiple comparisons to DTT plots as we primarily used them to identify the most divergent traits among the studied species. For these traits, we then plotted the trends in the highland and lowland species to explore which of the two drove the divergence in the whole dataset.

Finally, we tested for directional changes in trait values among willow species from the root of the phylogeny by correlating Abouheif's distance (distance from the root) with trait values, as calculated in the R package 'adephylo' (Jombart et al., 2010). We corrected the results for false discovery rate. If we discovered a significant correlation in the whole dataset, we also correlated Abouheif's distance with trait values for lowland and highland species, to examine which group drove the significant trend.

We included *Populus tremula* in multivariate and LIPA analyses to provide a broader context to the variation among willow species and in DTT plots to provide the same root and span for all plots. We excluded it from other analyses.

RESULTS

Herbivory damage did not differ between lowland and highland willows (f^2=0.027, $F_{(1,27)}$=0.72, p=0.405; Figure S2). The studied willow species showed high variation in most of the traits we used in PGLS analyses (Figure S2). Highland willow species were higher in concentration of flavonoids (SES=0.553, $F_{(1,27)}$=4.36, p=0.046) and had greater uniqueness in the whole metabolome (SES=0.711, $F_{(1,27)}$=7.21, p=0.012) while lowland species had greater uniqueness in salicinoids (SES=−0.797, $F_{(1,27)}$=9.01, p=0.006) (Figure 1, Table S5). The difference in concentration of flavonoids (p_{adj}=0.189) and in the two measures of uniqueness (p_{adj}=0.084 for both) became non-significant or marginally significant when we accounted for false discovery rate. Differences in other traits were not significant. Chemical composition of the whole metabolome in lowland and highland willow species largely overlapped when analysed with pPCA (Figure 2) and species in the two groups did not show significant difference in their CSCS distances ($t_{(23)}$=−1.73, p=0.092). When considering flavonoids, highland willows formed a subcluster within the lowland cluster, except for *Salix mielichhoferi*. Highland species exhibited lower CSCS distances in flavonoids than lowland species ($t_{(13)}$=3.84, p=0.002). Salicinoid composition only partly overlapped between lowland and highland species but CSCS distances in salicinoids were not significantly different between the two groups ($t_{(13)}$=−0.63, p=0.538).

We recovered significantly different phylogenetic trends in the chemistry of lowland and highland species.

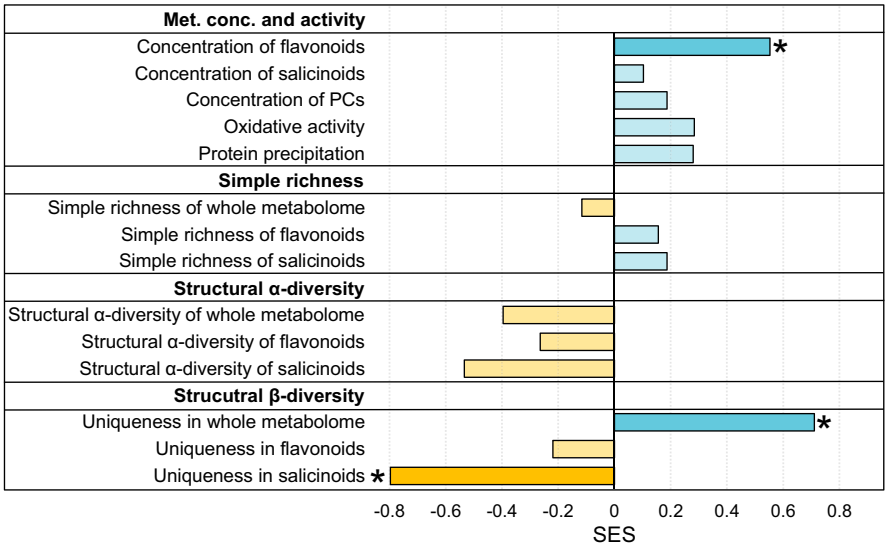

FIGURE 1 Standardised effect sizes (SES) extracted from PGLS models comparing highland and lowland willow species. Highland willow species were higher in concentration of flavonoids (SES=0.553, $F_{(1,27)}$=4.36, p=0.046) and uniqueness in the whole metabolome (SES=0.711, $F_{(1,27)}$=7.21, p=0.012) while lowland species were higher in uniqueness of salicinoids (SES=−0.797, $F_{(1,27)}$=9.01, p=0.006). The difference in concentration of flavonoids (p_{adj}=0.189) and in the two measures of uniqueness (p_{adj}=0.084 for both) became non-significant or marginally significant when we accounted for false discovery rate. Traits higher in highland willows are in blue and traits higher in lowland willows are in yellow.

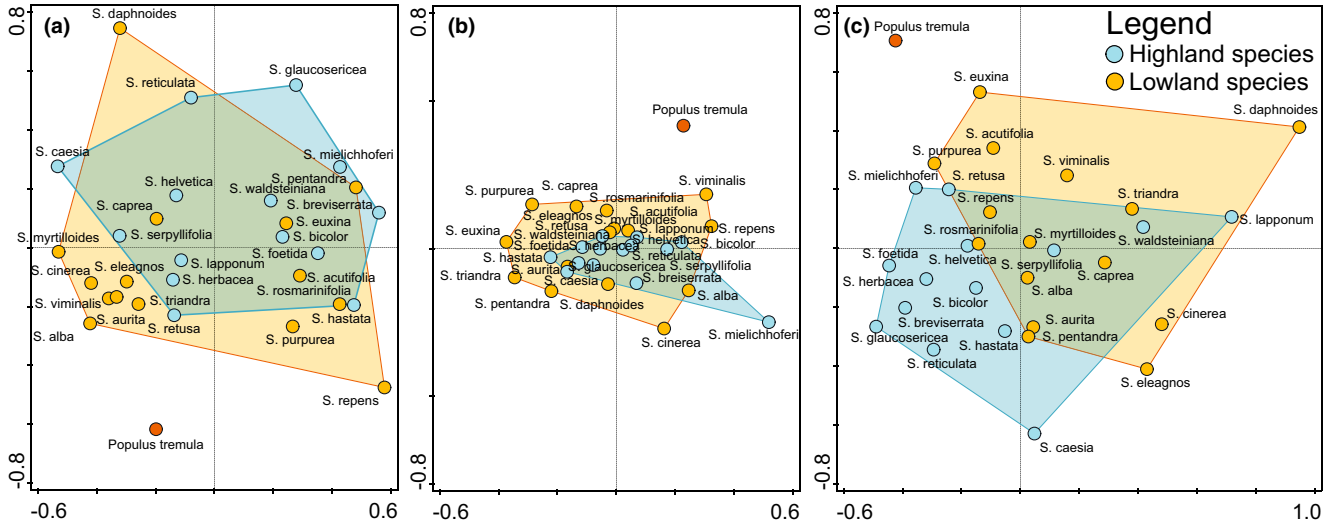

FIGURE 2 Partial Principal Component Analysis (pPCA) diagrams showing structural β-diversity in whole metabolome (a), flavonoids (b) and salicinoids (c) among lowland (yellow) and highland (blue) willow species. The β-diversity in willow chemistry was quantified with CSCS distance matrices that take structural similarity of individual metabolites into account to provide structural-compositional dissimilarity for individual samples. The first two pPCA axes explained 30.6% of the variation in whole metabolome, 39.9% of the variation in flavonoids and 32.9% of the variation in salicinoids. We used phylogenetic axes with significant effects on variation in willow chemistry as covariates to account for the effect of willow phylogeny.

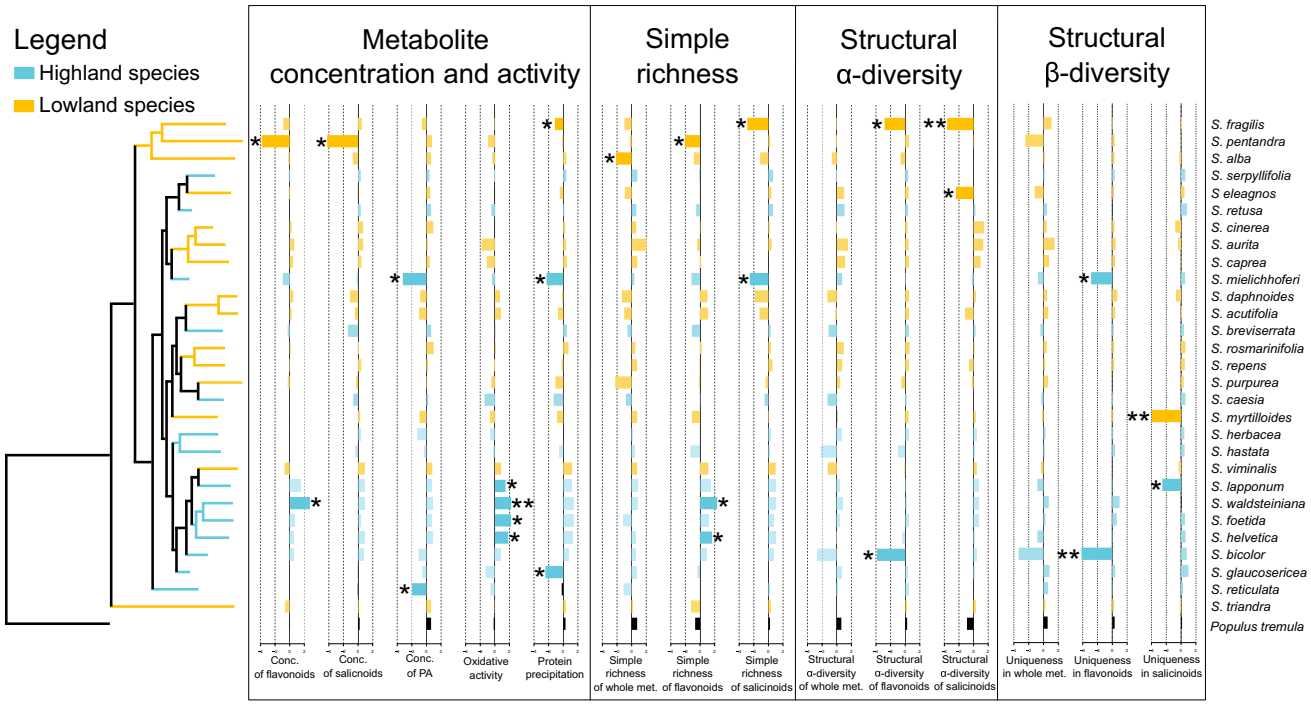

FIGURE 3 Deviations from local phylogenetic mean in the measured chemical traits. Columns pointing towards left suggest dissimilarity and divergence. Columns pointing towards right suggest similarity and conservatism. Lowland willows are in yellow and highland willows are in blue. Species with significant deviation in local Moran's index in the given trait are in darker colours and marked with one ($p<0.05$) or two ($p<0.01$) asterisks. All deviations except for deviation in uniqueness in flavonoids in *Salix bicolor* and uniqueness in salicinoids in *S. myrtilloides* became non-significant after correcting for false discovery rate.

When exploring the local phylogenetic means for individual traits, we found 19 significant deviations towards lower similarity and seven significant deviations towards higher similarity between related species (Figure 3). Most of the deviations, except for uniqueness in flavonoids in *Salix bicolor* and uniqueness in salicinoids in *S. myrtilloides*, became non-significant after correcting for false discovery rate. There was a greater number of deviations

towards higher similarity among highland willow species than among lowland willows species ($\chi^2_{(7)}=16.79$, $p=0.019$). The number of deviations towards lower similarity did not differ between the two datasets ($\chi^2_{(7)}=10.45$, $p=0.165$). When we explored divergence with DTT plots in the whole dataset, we identified flavonoid concentration, salicinoid concentration, proanthocyanidin concentration, simple richness of the whole metabolome, simple richness of salicinoids and structural α-diversity of flavonoids as traits showing divergence peaks outside the confidence intervals (Figure 4). The remaining eight traits did not show such a trend (Figure S3). The trends in flavonoid concentration, proanthocyanidin concentration, simple richness of salicinoids and structural α-diversity of flavonoids were driven by the lowland species. The concentration of salicinoids diverged in the highland species but the divergence peak occurred at a shallower level than in the whole dataset. None of the separate datasets showed a significant divergence in the case of simple richness of the whole metabolome.

Five out of the 14 chemical traits showed significant directional phylogenetic trends among the studied species (Table S6). We detected an increase from the root to the tip of our phylogenetic tree in flavonoid concentration ($f^2=0.447$, $F_{(1,27)}=12.07$, $p=0.002$), polyphenol oxidative activity ($f^2=0.538$, $F_{(1,27)}=14.51$, $p=0.001$), protein precipitation capacity ($f^2=0.157$, $F_{(1,27)}=4.25$, $p=0.049$), and simple richness of flavonoids ($f^2=0.553$, $F_{(1,27)}=14.93$, $p=0.001$). We detected a decrease in flavonoid uniqueness ($f^2=0.227$, $F_{(1,27)}=6.13$, $p=0.019$). While the other trends remained significant, the trends in protein precipitation and flavonoid uniqueness became non-significant after correcting for false discovery rate (Table S6). The significant trends in the whole dataset were driven by a strong increase in flavonoid concentration ($f^2=0.971$, $F_{(1,12)}=11.66$, $p=0.005$), polyphenol oxidative activity ($f^2=1.537$, $F_{(1,12)}=18.44$, $p=0.001$) and simple richness of flavonoids ($f^2=1.211$, $F_{(1,12)}=14.54$, $p=0.002$) among derived highland species (Figure 5).

DISCUSSION

Diversity of specialised metabolites helps plants cope with various biotic and abiotic pressures (Wetzel & Whitehead, 2020). Our results indicate that fundamental ecological and evolutionary pathways to chemical diversity vary across selective regimes experienced by wider communities of plants growing at different elevations.

We show that differences in chemistry among willows growing at different elevations occur mainly through shifts in chemical β-diversity. The differences in structural β-diversity between lowland and highland willows were most pronounced in flavonoids that serve plants as anti-oxidants and protection against abiotic factors such as UV-irradiation (Tegelberg & Julkunen-Tiitto, 2001;

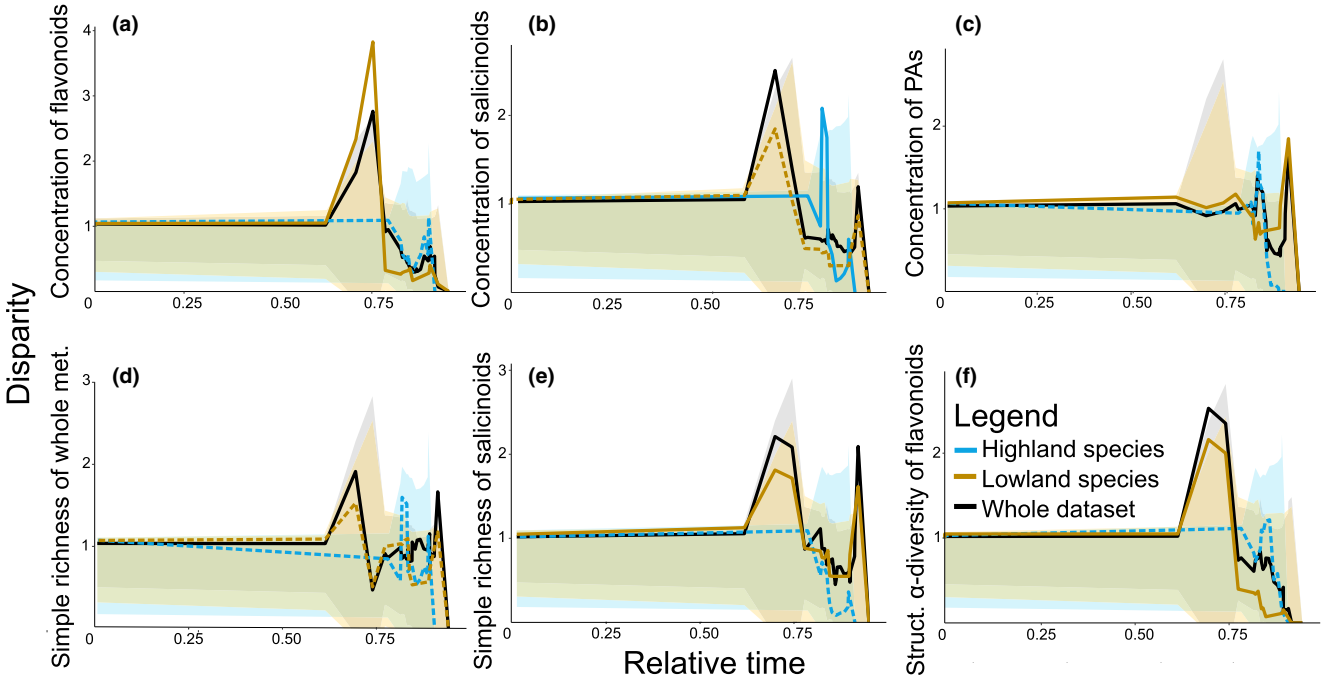

FIGURE 4 Mean disparity through time (DTT) plots for traits with significant deviation from character evolution expected under Brownian motion in the whole dataset. Solid and dashed lines show disparity in concentration of flavonoids (a), concentration of salicinoids concentration (b), concentration of proanthocyanidins (c), simple richness of whole metabolome (d), simple richness of salicinoids (e) and structural α-diversity of flavonoids (f). The solid lines show deviations from Brownian motion peaking outside the confidence intervals in the whole dataset (black), lowland willows (yellow) and highland willows (blue). Dashed lines show non-significant trends. The shaded areas indicate the 95% confidence interval for the simulated data based on 999 simulations of character evolution on the phylogeny of studied willow species under Brownian motion. We included *Populus tremula* in DTT plots to provide the same root and span for all plots.

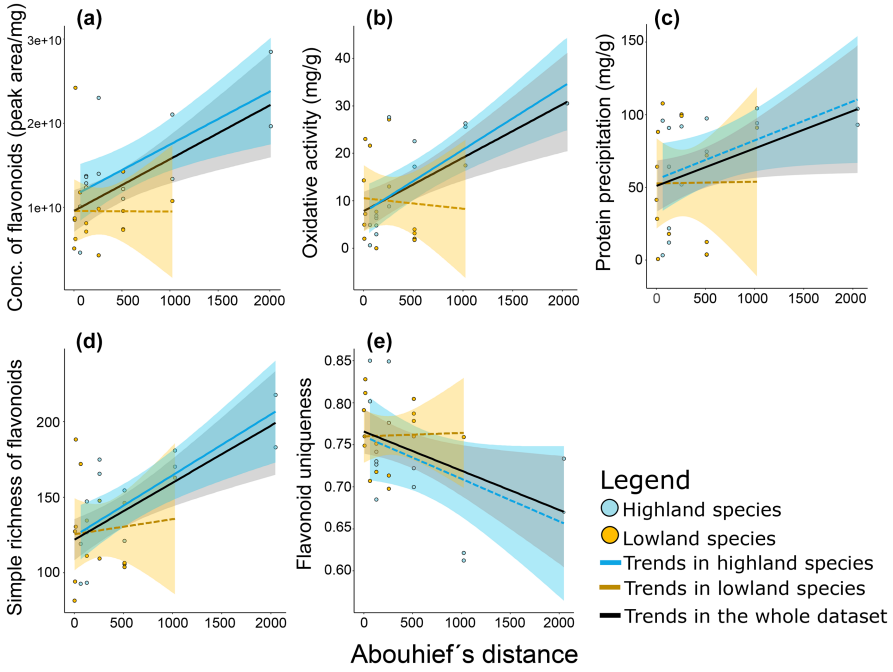

FIGURE 5 Scatter plots for traits showing significant correlation with distance from the root (Abouheif's distance) of the phylogenetic tree in the whole dataset. Flavonoid concentration (A; $F_{(1,27)}=12.07$, $p=0.002$), polyphenol oxidative activity (B; $F_{(1,27)}=14.51$, $p=0.001$), protein precipitation capacity (C; $F_{(1,27)}=4.25$, $p=0.049$), and simple richness of flavonoids (D; $F_{(1,27)}=14.93$, $p=0.001$) increased with the distance from the root of the tree including all willow species. Flavonoid uniqueness decreased (E; $F_{(1,27)}=6.13$, $p=0.019$). The trends in whole dataset were driven by increase in flavonoid concentration ($F_{(1,12)}=11.66$, $p=0.005$), polyphenol oxidative activity ($F_{(1,12)}=18.44$, $p=0.001$) and simple richness of flavonoids ($F_{(1,12)}=14.54$, $p=0.002$) among derived species of highland willows. The trends in protein precipitation and flavonoid uniqueness were no longer significant when we accounted for false discovery rate, and neither were significant these trends in the partial datasets. Black lines show trends in the whole dataset, yellow lines show trends in lowland species and blue lines show trends in highland species. The solid lines show significant trends. Dashed lines show non-significant trends. The shaded areas show the confidence intervals.

Treutter, 2006). In addition to showing lower structural β-diversity of flavonoids, highland species also showed more frequent phylogenetic deviations towards increased chemical similarity. These trends are consistent with previous studies reporting habitat filtering in plant assemblages mediated by traits associated with abiotic tolerance (Bakhtiari et al., 2021; Savage & Cavender-Bares, 2012; Sedio et al., 2012). Willows have repeatedly colonised alpine habitats and the focal highland species have probably evolved similar flavonoid profiles independently (Wagner et al., 2020). Additionally, our results suggest that highland willows have converged towards structurally similar flavonoids, possibly producing only those with the highest bioactivity relative to concentration (Volf et al., 2022). This may be especially important given the shorter alpine growing season and resource-poor habitat, perhaps explaining why many plants from high elevations invest in high concentrations of bioactive metabolites (Defossez et al., 2021).

Our results also point to differential elevational clines in β-diversity of various metabolites that likely depend on their importance for survival at high elevations. In contrast to our first hypothesis, we found a marginally higher β-diversity in the whole metabolome of highland species. These results are similar to Volf et al. (2022), who found an elevational increase in intra- and interspecific variation in overall willow metabolomes. The whole metabolome includes metabolites with diverse functions, some of which are possibly less essential to survival at high elevations. Such metabolites may be more prone to random changes due to detrimental effects of abiotic factors on metabolic pathways (Pellissier et al., 2014). Changes in the whole metabolome, including primary metabolites, may also reflect different microhabitat preferences of high elevation species (Volf et al., 2022).

We did not find significant differences in concentration and α-diversity of metabolites between highland and lowland willow species. The absence of significant differences in metabolite concentration and α-diversity observed in the studied species may have resulted from recent postglacial colonisation of the Alps from different ice-free refugia (Wagner et al., 2021), which could blur any macroevolutionary patterns. Although our limited taxonomic sampling prevents us from inferring macroevolutionary patterns more robustly, we found differential phylogenetic trends among highland and lowland species. These trends might be associated with changes in elevational range and may indicate differential evolutionary trajectories in chemistry between species growing under different selection pressures (Maron et al., 2019; Volf et al., 2019).

Contrastingly to our expectations, we observed a directional trend towards a higher simple richness of flavonoids among phylogenetically derived highland willow species. Similar phylogenetic trends may underline the high chemical α-diversity in highland plants reported by recent studies (Bakhtiari et al., 2021; Defossez et al., 2021; Moreira et al., 2018; Volf et al., 2020). It is difficult to assess whether producing a greater richness of structurally related metabolites can provide high-elevation plants with ecological benefits, for example, through positive interactions between individual metabolites (Wetzel & Whitehead, 2020). In another study, we found a decrease in structural α-diversity of flavonoids within willows growing along an elevational gradient (Volf et al., 2022). Together, these results suggest that harsh abiotic conditions may favour production of related metabolites through small tweaks in metabolic pathways, while reducing production of structurally distinct metabolites in willows.

We based several of our expectations on the fact that damage from herbivores almost invariably decreases towards higher elevations when considering single continuous elevational gradients (Pellissier et al., 2014; Sam et al., 2019; Volf et al., 2022). Here we observed similar levels of herbivory on highland and lowland willows. This may occur due to a hump-shaped elevational cline in herbivory, variation in herbivore community composition at the regional scale or seasonal accumulation of herbivory that were not captured by our measurements (Landsberg & Gillieson, 1995). Nevertheless, the comparatively uniform levels of overall herbivory in lowland and highland willows may be consistent with the phylogenetic trends in concentration of chemical defences due to underlying gradients in abiotic stress (Defossez et al., 2018; Moreira et al., 2018). For example, it could explain the trend towards higher oxidative activity among derived highland species that need to defend their valuable biomass, having limited resources for compensatory regrowth (Pellissier et al., 2016; Salgado et al., 2016). Production of reactive radicals through polyphenol oxidative activity has pronounced effects on insect herbivores (Salminen & Karonen, 2011), and these may be elevated at high elevations where herbivores face oxidative stress due to abiotic conditions. However, the comparative levels of herbivory do not explain the chemical divergence or marginally higher β-diversity in salicinoids we observed in lowland willows.

The effect of antagonistic biotic interactions on the traits that characterise plant communities is thought to vary with the host specificity of plant enemies (Volf et al., 2018). Simulation models suggest that enemies of intermediate host specificity favour local assemblages of the most chemically distinct plant species (Sedio & Ostling, 2013). This is because chemical divergence in local communities filtered from the regional pool allows plants to avoid sharing their oligophagous herbivores (Becerra, 2007; Kursar et al., 2009; Volf et al., 2019). Willows harbour a diverse selection of oligophagous herbivores feeding only on Salicaceae (Hjalten et al., 2007; Volf et al., 2015). We lack herbivore community data and cannot compare herbivore host specificity between lowland and highland willows. Some specialised herbivores, such as sawflies that use salicinoids as feeding cues, are cold-adapted and can occur at relatively high elevations (Hjalten et al., 2007; Nyman et al., 2006). This could explain the divergence in salicinoid concentration among highland willow species. However, sawflies are typically less abundant than other specialised herbivores in the region of our study (Volf et al., 2015). In light of our phytochemical results, we therefore predict that willow-herbivore host specificity declines with elevation as it does in other plant systems in the region (Pellissier et al., 2012) and may be one reason for more frequent chemical divergence among the lowland species. Further studies on elevational clines in herbivore community composition are thus needed to explore if the observed differences between highland and lowland willows are driven by elevational variation in the host specificity of herbivores.

Overall, we show that different trends contribute to distinct aspects of chemical diversity in speciose genera of plants occupying different realised habitats. While abiotic stress at high elevations filters plants towards similar chemistry and possibly supports chemical convergence, lowland conditions promote divergence between closely related species. Differential trends in alternative measures of chemical diversity among lowland and highland plants may shed light on trends in plant chemistry along other major ecological gradients. The hypothesis that biotic interactions are more intense in the tropics and contribute to the global latitudinal diversity gradient has been popular since Wallace (1878) and Dobzhansky (1950). However, evaluations of this prediction that have focused on overall rates of herbivory and variation in concentration of broad chemical classes have found either little variation with latitude or greater plant investment in defence at higher latitudes (Moles et al., 2011). Contrastingly to coarse herbivory rates, herbivore host specialisation does appear to decrease with latitude at the global scale (Dyer et al., 2007). Similarly, chemical β-diversity appears to be greater in warmer climates (Sedio et al., 2021) and tropical latitudes (Sedio, Parker, et al., 2018). These latitudinal patterns of herbivory, host specialisation, quantitative investment in chemical defence and chemical β-diversity may be analogous to elevational clines due to similar underlying gradients in the relative importance of abiotic stress and biotic interactions (Becerra, 2015). The rapidly expanding field of metabolomics promises to reveal the mechanisms by which plant interactions with their biotic and abiotic environment govern and are governed by convergence or divergence in various aspects of plant chemical diversity along major ecological gradients (Philbin et al., 2022; Sedio, Parker, et al., 2018).

AUTHOR CONTRIBUTIONS

MV designed the general approach and proposed the hypotheses; MV, TV and PK collected the data following the advice of EH; MV, EH and NDW identified the plants; NL, J-PS and BES conducted the chemical analysis; PdLF inferred the phylogeny with support of JVL, PM-M and NDW; MV, JVL and STS conducted the statistical analyses; MV wrote the first draft of the manuscript; all authors critically contributed to the final draft.

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PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ele.14273.

DATA AVAILABILITY STATEMENT

The data on studied willows, their traits, sequence alignments, consensus sequence alignments and the phylogenetic trees are available from the Zenodo Repository (https://doi.org/10.5281/zenodo.7825538). The genomics data are available from the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra), SRA: SRP344656. All command and parameters used to infer the phylogeny can be found in the WGS of willows documentation on our GitHub page (https://github.com/paola-ferreira/WGS-of-willows). The metabolomics data are available as a MassIVE dataset, MSV000091773, from https://massive.ucsd.edu/. The code for statistical analyses performed in R is available from the Zenodo Repository (https://doi.org/10.5281/zenodo.7984460).

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REFERENCES

Abràmoff, M.D., Magalhães, P.J. & Ram, S.J. (2004) Image processing with ImageJ. *Biophotonics International*, 11, 36–42.
Bakhtiari, M., Glauser, G., Defossez, E. & Rasmann, S. (2021) Ecological convergence of secondary phytochemicals along elevational gradients. *New Phytologist*, 229, 1755–1767.
Becerra, J.X. (2007) The impact of herbivore-plant coevolution on plant community structure. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 7483–7488.
Becerra, J.X. (2015) On the factors that promote the diversity of herbivorous insects and plants in tropical forests. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 6098–6103.
Benjamini, Y. & Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, 57, 289–300.
Cavender-Bares, J., Ackerly, D.D., Baum, D. & Bazzaz, F. (2004) Phylogenetic overdispersion in Floridian oak communities. *The American Naturalist*, 163, 823–843.
Chambers, J.M. & Hastie, T.J. (2017) Statistical models. In: *Statistical models in S*. New York: Routledge, pp. 13–44.
Defossez, E., Pellissier, L. & Rasmann, S. (2018) The unfolding of plant growth form-defence syndromes along elevation gradients. *Ecology Letters*, 21, 609–618.
Defossez, E., Pitteloud, C., Descombes, P., Glauser, G., Allard, P.-M., Walker, T.W. et al. (2021) Spatial and evolutionary predictability of phytochemical diversity. *Proceedings of the National Academy of Sciences of the United States of America*, 118, e2013344118.
Denno, R.F., Larsson, S. & Olmstead, K.L. (1990) Role of enemy-free space and plant quality in host-plant selection by willow beetles. *Ecology*, 71, 124–137.
Dobzhansky, T. (1950) Evolution in the tropics. *American Scientist*, 38, 209–221.
Dührkop, K., Fleischauer, M., Ludwig, M., Aksenov, A.A., Melnik, A.V., Meusel, M. et al. (2019) SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. *Nature Methods*, 16, 299–302.
Dührkop, K., Nothias, L.-F., Fleischauer, M., Reher, R., Ludwig, M., Hoffmann, M.A. et al. (2021) Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. *Nature Biotechnology*, 39, 462–471.
Dührkop, K., Shen, H., Meusel, M., Rousu, J. & Böcker, S. (2015) Searching molecular structure databases with tandem mass spectra using CSI: FingerID. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 12580–12585.

Dyer, L.A., Singer, M.S., Lill, J.T., Stireman, J.O., Gentry, G.L., Marquis, R.J. et al. (2007) Host specificity of lepidoptera in tropical and temperate forests. *Nature*, 448, 696–U699.

Ehrlich, P.R. & Raven, P.H. (1964) Butterflies and plants—a study in coevolution. *Evolution*, 18, 586–608.

Engström, M., Arvola, J., Nenonen, S., Virtanen, V., Leppa, M., Tahtinen, P. et al. (2019) Structural features of hydrolyzable tannins determine their ability to form insoluble complexes with bovine serum albumin. *Journal of Agricultural and Food Chemistry*, 67, 6798–6808.

Engström, M.T., Pälijärvi, M., Fryganas, C., Grabber, J.H., Mueller-Harvey, I. & Salminen, J.-P. (2014) Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, 62, 3390–3399.

Engström, M.T., Pälijärvi, M. & Salminen, J.-P. (2015) Rapid fingerprint analysis of plant extracts for ellagitannins, gallic acid, and quinic acid derivatives and quercetin-, kaempferol- and myricetin-based flavonol glycosides by UPLC-QqQ-MS/MS. *Journal of Agricultural and Food Chemistry*, 63, 4068–4079.

Harmon, L.J., Weir, J.T., Brock, C.D., Glor, R.E. & Challenger, W. (2008) GEIGER: investigating evolutionary radiations. *Bioinformatics*, 24, 129–131.

He, L., Wagner, N.D. & Hörandl, E. (2021) Restriction-site associated DNA sequencing data reveal a radiation of willow species (L., Salicaceae) in the Hengduan Mountains and adjacent areas. *Journal of Systematics and Evolution*, 59, 44–57.

Hjalten, J., Niemi, L., Wennstrom, A., Ericson, L., Roininen, H. & Julkunen-Tiitto, R. (2007) Variable responses of natural enemies to *Salix triandra* phenotypes with different secondary chemistry. *Oikos*, 116, 751–758.

Jombart, T., Balloux, F. & Dray, S. (2010) Adephylo: new tools for investigating the phylogenetic signal in biological traits. *Bioinformatics*, 26, 1907–1909.

Karger, D.N. & Zimmermann, N.E. (2019) *Climatologies at high resolution for the earth land surface areas CHELSA V1. 2: technical specification*. Switzerland: Swiss Federal Research Institute WSL.

Keck, F., Rimet, F., Bouchez, A. & Franc, A. (2016) Phylosignal: an R package to measure, test, and explore the phylogenetic signal. *Ecology and Evolution*, 6, 2774–2780.

Kursar, T.A., Dexter, K.G., Lokvam, J., Pennington, R.T., Richardson, J.E., Weber, M.G. et al. (2009) The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 18073–18078.

Landsberg, J. & Gillieson, D. (1995) Regional and local variation in insect herbivory, vegetation and soils of eucalypt associations in contrasted landscape positions along a climatic gradient. *Australian Journal of Ecology*, 20, 299–315.

Lenth, R., Singmann, H., Love, J., Buerkner, P. & Herve, M. (2019) Package 'emmeans'.

Malisch, C.S., Salminen, J.-P., Kölliker, R., Engström, M.T., Suter, D., Studer, B. et al. (2016) Drought effects on proanthocyanidins in sainfoin (*Onobrychis viciifolia* Scop.) are dependent on the plant's ontogenetic stage. *Journal of Agricultural and Food Chemistry*, 64, 9307–9316.

Maron, J.L., Agrawal, A.A. & Schemske, D.W. (2019) Plant-herbivore coevolution and plant speciation. *Ecology*, 100, e02704.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., Von Haeseler, A. et al. (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution*, 37, 1530–1534.

Moles, A.T., Bonser, S.P., Poore, A.G.B., Wallis, I.R. & Foley, W.J. (2011) Assessing the evidence for latitudinal gradients in plant defence and herbivory. *Functional Ecology*, 25, 380–388.

Moreira, X., Petry, W.K., Mooney, K.A., Rasmann, S. & Abdala-Roberts, L. (2018) Elevational gradients in plant defences and insect herbivory: recent advances in the field and prospects for future research. *Ecography*, 41, 1485–1496.

Ning, D., Yuan, M., Wu, L., Zhang, Y., Guo, X., Zhou, X. et al. (2020) A quantitative framework reveals ecological drivers of grassland microbial community assembly in response to warming. *Nature Communications*, 11, 1–12.

Nothias, L.-F., Petras, D., Schmid, R., Dührkop, K., Rainer, J., Sarvepalli, A. et al. (2020) Feature-based molecular networking in the GNPS analysis environment. *Nature Methods*, 17, 905–908.

Nyman, T., Farrell, B.D., Zinovjev, A.G. & Vikberg, V. (2006) Larval habits, host-plant associations, and speciation in nematine sawflies (Hymenoptera: Tenthredinidae). *Evolution*, 60, 1622–1637.

Paradis, E. & Schliep, K. (2019) Ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, 35, 526–528.

Pellissier, L., Descombes, P., Hagen, O., Chalmandrier, L., Glauser, G., Kergunteuil, A. et al. (2018) Growth-competition-herbivore resistance trade-offs and the responses of alpine plant communities to climate change. *Functional Ecology*, 32, 1693–1703.

Pellissier, L., Fiedler, K., Ndribe, C., Dubuis, A., Pradervand, J.N., Guisan, A. et al. (2012) Shifts in species richness, herbivore specialization, and plant resistance along elevation gradients. *Ecology and Evolution*, 2, 1818–1825.

Pellissier, L., Moreira, X., Danner, H., Serrano, M., Salamin, N., van Dam, N.M. et al. (2016) The simultaneous inducibility of phytochemicals related to plant direct and indirect defences against herbivores is stronger at low elevation. *Journal of Ecology*, 104, 1116–1125.

Pellissier, L., Roger, A., Bilat, J. & Rasmann, S. (2014) High elevation *Plantago lanceolata* plants are less resistant to herbivory than their low elevation conspecifics: is it just temperature? *Ecography*, 37, 950–959.

Philbin, C.S., Dyer, L.A., Jeffrey, C.S., Glassmire, A.E. & Richards, L.A. (2022) Structural and compositional dimensions of phytochemical diversity in the genus piper reflect distinct ecological modes of action. *Journal of Ecology*, 110, 57–67.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & Team, R.D.C. (2019) Nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-140 https://CRAN.R-project.org/package=nlme

Pluskal, T., Castillo, S., Villar-Briones, A. & Orešič, M. (2010) MZmine 2: modular framework for processing, visualizing, and analyzing massspectrometry-based molecular profile data. *BMC Bioinformatics*, 11, 1–11.

R Core Team. (2022) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org

Revell, L.J. (2012) Phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, 3, 217–223.

Salgado, A.L., Suchan, T., Pellissier, L., Rasmann, S., Ducrest, A.-L. & Alvarez, N. (2016) Differential phenotypic and genetic expression of defence compounds in a plant–herbivore interaction along elevation. *Royal Society Open Science*, 3, 160226.

Salminen, J.P. & Karonen, M. (2011) Chemical ecology of tannins and other phenolics: we need a change in approach. *Functional Ecology*, 25, 325–338.

Sam, K., Koane, B., Sam, L., Mrazova, A., Segar, S.T., Volf, M. et al. (2019) Insect herbivory and herbivores of *Ficus* species along a rainforest elevational gradient in Papua New Guinea. *Biotropica*, 52, 263–276.

Savage, J.A. & Cavender-Bares, J. (2012) Habitat specialization and the role of trait lability in structuring diverse willow (genus *Salix*) communities. *Ecology*, 93, S138–S150.

Sedio, B.E., Boya, P.C.A. & Rojas Echeverri, J.C. (2018) A protocol for high-throughput, untargeted forest community metabolomics using mass spectrometry molecular networks. *Applications in Plant Sciences*, 6, e1033.

Sedio, B.E. & Ostling, A.M. (2013) How specialised must natural enemies be to facilitate coexistence among plants? *Ecology Letters*, 16, 995–1003.

Sedio, B.E., Parker, J.D., McMahon, S.M. & Wright, S.J. (2018) Comparative foliar metabolomics of a tropical and a temperate forest community. *Ecology*, 99, 2647–2653.
Sedio, B.E., Rojas Echeverri, J.C., Boya, P., Cristopher, A. & Wright, S.J. (2017) Sources of variation in foliar secondary chemistry in a tropical forest tree community. *Ecology*, 98, 616–623.
Sedio, B.E., Spasojevic, M.J., Myers, J.A., Wright, S.J., Person, M.D., Chandrasekaran, H. et al. (2021) Chemical similarity of co-occurring trees decreases with precipitation and temperature in North American forests. *Frontiers in Ecology and Evolution*, 9, 679638.
Sedio, B.E., Wright, S.J. & Dick, C.W. (2012) Trait evolution and the coexistence of a species swarm in the tropical forest understorey. *Journal of Ecology*, 100, 1183–1193.
Tegelberg, R. & Julkunen-Tiitto, R. (2001) Quantitative changes in secondary metabolites of dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced ultraviolet-B radiation. *Physiologia Plantarum*, 113, 541–547.
ter Braak, C.J. & Smilauer, P. (2012) Canoco Reference Manual and User's Guide: Software for Ordination (version 5.0). Microcomputer power, Ithaca.
Treutter, D. (2006) Significance of flavonoids in plant resistance: a review. *Environmental Chemistry Letters*, 4, 147–157.
Tripathi, A., Vázquez-Baeza, Y., Gauglitz, J.M., Wang, M., Dührkop, K., Nothias-Esposito, M. et al. (2021) Chemically informed analyses of metabolomics mass spectrometry data with Qemistree. *Nature Chemical Biology*, 17, 146–151.
Volf, M., Hrcek, J., Julkunen-Tiitto, R. & Novotny, V. (2015) To each its own: differential response of specialist and generalist herbivores to plant defence in willows. *Journal of Animal Ecology*, 84, 1123–1132.
Volf, M., Laitila, J.E., Kim, J., Sam, L., Sam, K., Isua, B. et al. (2020) Compound specific trends of chemical Defences in *Ficus* along an elevational gradient reflect a complex selective landscape. *Journal of Chemical Ecology*, 46, 442–454.
Volf, M., Salminen, J.-P. & Segar, S.T. (2019) Evolution of defences in large tropical plant genera: perspectives for exploring insect diversity in a tri-trophic context. *Current Opinion in Insect Science*, 32, 91–97.
Volf, M., Segar, S.T., Miller, S.E., Isua, B., Sisol, M., Aubona, G. et al. (2018) Community structure of insect herbivores is driven by conservatism, escalation and divergence of defensive traits in *Ficus*. *Ecology Letters*, 21, 83–92.
Volf, M., Volfová, T., Hörandl, E., Wagner, N.D., Luntamo, N., Salminen, J.P. et al. (2022) Abiotic stress rather than biotic interactions drives contrasting trends in chemical richness and variation in alpine willows. *Functional Ecology*, 36, 2701–2712.
Wagner, N.D., He, L. & Hörandl, E. (2020) Phylogenomic relationships and evolution of polyploid *Salix* species revealed by RAD sequencing data. *Frontiers in Plant Science*, 11, 1077.
Wagner, N.D., He, L. & Hörandl, E. (2021) The evolutionary history, diversity, and ecology of willows (*Salix* L.) in the European Alps. *Diversity*, 13, 146.
Wallace, A.R. (1878) *Tropical nature, and other essays*. London: Macmillan and Company.
Wang, M., Carver, J.J., Phelan, V.V., Sanchez, L.M., Garg, N., Peng, Y. et al. (2016) Sharing and community curation of mass spectrometry data with global natural products social molecular networking. *Nature Biotechnology*, 34, 828.
Webb, C.O., Ackerly, D.D., McPeek, M.A. & Donoghue, M.J. (2002) Phylogenies and community ecology. *Annual Review of Ecology and Systematics*, 33, 475–505.
Wetzel, W.C. & Whitehead, S.R. (2020) The many dimensions of phytochemical diversity: linking theory to practice. *Ecology Letters*, 23, 16–32.
Whitehead, S.R., Bass, E., Corrigan, A., Kessler, A. & Poveda, K. (2021) Interaction diversity explains the maintenance of phytochemical diversity. *Ecology Letters*, 24, 1205–1214.
Zhou, R., Macaya-Sanz, D., Carlson, C.H., Schmutz, J., Jenkins, J.W., Kudrna, D. et al. (2020) A willow sex chromosome reveals convergent evolution of complex palindromic repeats. *Genome Biology*, 21, 1–19.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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